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(54) Title: ASSAY FOR ASSEMBLY OF HERPES SIMPLEX VIRUS CAPSID

(57) Abstract

The present invention relates to a method of assembling HSV capsids *in vitro*. The method of assembling HSV capsids can be used to screen for compositions that inhibit the assembly of HSV capsids. In addition the *in vitro* synthesized capsids can be used as delivery vehicles for delivering pharmaceutical agents to animals.

Assay for Assembly of Herpes Simplex Virus Capsid

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Field of the Invention

The present invention is directed to a system for assembling virus

10   capsids *in vitro* and the use of such a system as an assay for therapeutics that are capable of interfering with capsid formation. In addition, the capsids formed *in vitro* can be utilized as delivery vehicles for the oral delivery of pharmaceutical compositions to the intestines of vertebrates.

15   ### Background of the Invention

Herpes simplex virus type 1 (HSV-1) is well-known as the etiological agent of cold sores, venereal lesions and neonatal encephalitis. Like all herpes viruses, the HSV-1 virion consists of an icosahedral capsid surrounded by a membrane envelope. The viral dsDNA is contained inside the capsid while a layer of

20   protein called the tegument is found between the capsid and the membrane. The mature capsid is an icosahedral protein shell approximately 15 nm thick and 126 nm in diameter. Its principal structural features are 162 capsomers (150 hexons and 12 pentons) that lie on a T = 16 icosahedral lattice. Each capsomer consists of a roughly cylindrical protruding domain that is extended laterally at its proximal end to create

25   the capsid floor layer (3 nm- 4 nm in thickness). The floor is the only place where capsomers make direct contact with each other. Capsomers, however, are also connected indirectly by way of the triplexes, trigonal structures (320 in all) that lie above the floor layer with one triplex found at the local three-fold position created by each group of three capsomers.

30   VP5, the HSV-1 major capsid protein (MW 149,075), is the predominant polypeptide component of the capsid; it is the structural subunit of both the hexons (hexamers of VP5) and pentons (pentamers of VP). The triplexes are

composed of two minor capsid proteins, VP19C and VP23. Most, if not all, triplexes contain one molecule of VP19C and two of VP23.

HSV-1 capsids are formed in the infected-cell nucleus where they are also packaged with DNA prior to further virus maturation. Assembly requires the three capsid structural proteins mentioned above plus a scaffolding protein. In cells infected with wild type (wt) HSV-1, the primary scaffolding protein is pre-VP22a (also called ICP35; the product of the UL26.5 gene), although VP21, a cleavage product of the polypeptide encoded by UL26, can also serve effectively as a scaffolding protein. During capsid assembly the scaffolding protein binds to VP5 and forms a core internal to the shell proteins. Pre- VP22a is cleaved to VP22a and exits the capsid at or near the time DNA enters and is not found in the mature virion.

Intermediates in the capsid assembly process have been identified in studies involving use of a cell-free assembly system. The system is based on use of a panel of recombinant baculoviruses (rBV) encoding HSV-1 capsid proteins. It is constituted by mixing extracts of rBV-infected insect cells containing HSV-1 proteins and incubating the mixture *in vitro*. Studies with the system have demonstrated that capsids are formed by way of partial and complete procapsid intermediates. Partial procapsids are arc- or dome-shaped structures in which a region of capsid shell partially surrounds a region of core. They grow into complete procapsids as the shell is enlarged and closed. The procapsid has T = 16 icosahedral symmetry and the same diameter as the mature capsid, but the two differ in several important ways. First, procapsids are spherical in shape while the mature capsid is icosahedral. Second, the floor layer in the procapsid is not continuous resulting in a structure that has large gaps between the capsomers. No comparable gaps are found in the mature capsid floor layer. Third, hexons are oval-shaped in the procapsid rather than hexagonal as they are in the mature form. Lastly, the procapsid is a more fragile structure than the mature capsid. For example, the procapsid is disassembled during incubation at 2°C, a treatment that does not affect the integrity of mature capsids.

Although the cell-free system described above has been employed productively to clarify features of the assembly, and the system can be used to form procapsids *in vitro*, this system is not optimal for investigating potential therapeutics that interfere with capsid assembly nor can it be used to create a pharmaceutical

delivery vehicle. The variability of the cell extract component makes studies of compounds that inhibit procapsid formation problematic and the presence of insect cell proteins and other cell extract components prevent the current *in vitro* assemble systems from being used as an efficient pharmaceutical delivery vehicles.

The presently described *in vitro* synthesis procedure provides a well defined *in vitro* assembly system that uses highly purified components. Thus the system can be used to test a wide variety of compounds for their ability to inhibit capsid formation while allowing a direct comparison from one test to another. In addition, the present system provides a sufficiently characterized system that ensures that no extraneous cellular proteins are encapsulated and that no infective HSV-1 viruses are formed during the process. Thus the *in vitro* assembly system provides a safe and effective delivery vehicle for the oral delivery of protease and pH sensitive pharmaceuticals.

Summary of the Invention

An *in vitro* system is described for the assembly of herpes simplex virus (HS V-1) procapsids beginning with three purified components, the major capsid protein (VPS), the triplexes (VP19C plus VP23) and a scaffolding protein. Each component is purified from insect cells expressing the relevant protein(s) from an appropriate recombinant baculovirus vector. Procapsids formed when the three purified components were mixed and incubated for 1 hr at 37°C.

Brief Description of the Drawings

Fig. 1 is a schematic drawing showing the steps in the formation of the herpes simplex virus capsid.

Fig. 2 is a schematic drawing showing one protocol for using *in vitro* HSV-1 capsid assembly to introduce therapeutics into the capsid. A partial procapsid is formed first followed by the assembly of the procapsid and then the procapsid undergoes a structural transformation to form a mature capsid.

Fig. 3A is a schematic drawing showing an alternative protocol for using *in vitro* HSV-1 capsid assembly to introduce therapeutics into the capsid. This protocol uses a hybrid scaffold protein that is bound to a cargo molecule (such as a

therapeutic agent). A partial procapsid is formed first followed by the assembly of the procapsid and then the procapsid undergoes a structural transformation to form a mature capsid.

Fig. 3B is a schematic drawing of one embodiment of a hybrid scaffolding protein with a cargo molecule (for example a therapeutic agent) linked to the scaffolding protein via a protease sensitive linker.

Detailed Description of the Invention

The HSV-1 virion consists of an icosahedral capsid surrounded by a membrane envelope. The principal morphological features of the capsid are 162 capsomers each of which is roughly cylindrical in shape (15 nm long by ~11 nm in diameter). Capsomers are arranged with their long axes projecting radially outward from the center of the capsid. Each capsomer is joined to its neighbors by extensions at its proximal end and also by trigonal structures called triplexes.

The capsomers are composed of the HSV-1 major capsid protein, VP5 (MW 149,075). Each triplex is a heterotrimer consisting of one VP19C (MW 50,260) and two VP23 (MW 34,268) molecules. In addition to the capsomer and triplex proteins, assembly of the HSV-1 capsid requires the scaffolding protein pre-VP22a (MW 33,760). Two scaffolding protein molecules are used for each VP5 incorporated into the capsid shell. The scaffolding protein is located inside the capsid shell.

Assembly of the capsid takes place as complexes of VP5 and pre-VP22a condense with triplexes to form first arc- or dome-shaped structures, which are completed to create an enclosed sphere called the procapsid. The procapsid then angularizes to form the mature icosahedral capsid in a morphological transformation that does not require further incorporation of capsid protein molecules. The pathway of HSV-1 capsid formation is illustrated in Fig. 1.

A cell-free system for assembling viral capsids *in vitro* has been previously described, however that system suffers from several disadvantages that prevent the system from being used to screen for bioactive agents that inhibit capsid formation or use of the system as a delivery vehicle. The greatest disadvantage of the previously described system is the fact that it uses cell extracts and thus, due to the variability of the cell extract component, the system is not fully characterized. In

order to create a more well defined *in vitro* assembly system for HSV-1 capsid formation, HSV-1 capsid proteins were purified biochemically from rBV-infected insect cells expressing them and used to assemble procapsids *in vitro*.

In accordance with one embodiment a procedure is described for forming viral capsids *in vitro* using a well defined mixture of purified proteins. In one preferred embodiment the viral capsid to be assembled *in vitro* is an HSV-1 viral capsid and four purified HSV-1 proteins, including VP5, VP23, VP19C and a viral scaffold protein, are combined in an aqueous solution and incubated for about one to about five hours at about 16°C to about 40°C. More preferably the mixture is incubated for about 1 to about 2 hours at 37°C.

The scaffolding protein can be the native HSV-1 scaffolding protein (pre-VP22a; UL26.5 gene) or a derivative thereof. In accordance with one embodiment, a hybrid scaffolding protein called pUL80.5-H (MW 39,855; see Oien, et al., J. Virol. 71:1281-1291 (1997)) is substituted for the native HSV-1 scaffolding protein for use in the *in vitro* assembly process. The pUL80.5-H protein consists of the N-terminal 364 amino acids of the human cytomegalovirus (HCMV) scaffolding protein (UL80.5 gene) linked to the C-terminal 25 amino acids of the corresponding HSV-1 protein (pre-VP22a; UL26.5 gene).

The purified proteins used in the *in vitro* assembly system of the present invention are isolated from recombinant cells that express one or more of the structural components of the viral capsid. In accordance with one preferred embodiment the VP5, scaffolding protein and triplexes (complexes of VP19C and VP23) used in the *in vitro* assembly process are purified separately by standard biochemical procedures after expressing those proteins using the baculovirus system. Specifically, the HSV-1 proteins including VP5, VP23, VP19C and a viral scaffold protein are expressed in insect (Sf9) cells as a result of infection with a recombinant baculovirus (rBV) vector or vectors encoding the required HSV-1 protein(s). In accordance with one embodiment, HSV-1 proteins VP5 and pUL80.5-H were purified from cells infected with BAC-UL19 and BAC-UL80.5-H, respectively, while triplexes were purified from cells co-infected with BAC-UL18 and BAC-UL38.

The method for preparing HSV-1 viral capsids *in vitro*, comprises combining four purified HSV-1 proteins including VP5, VP23, VP19C and a viral

scaffold protein in an aqueous solution and incubating the resulting mixture at about 16°C to about 40°C for about 0.5 to about 4 hours, more preferably about 1 to about 2 hours at 37°C. In one embodiment, procapsids were assembled in reaction mixtures containing about 10 ul VP5 (1-2 mg/ml), about 10 ul triplexes (-1 mg/ml) and 10

5   about ul pUL80.5-H (2-3 mg/ml). Typical reaction mixtures therefore contained approximately 70 pmoles VP5, 84 pmoles triplexes and 500 pmoles pUL80.5-H. Reaction mixtures were adjusted to 25 mM EDTA, 10 mM DTT, and protease inhibitors were added before incubation for 1 hr at 37°C. As capsid formation takes place the solution becomes increasingly more turbid and thus the incubation time for

10   forming capsids can be determined by measuring the turbidity of the mixture. After formation of the mature capsids the capsids are isolated using standard techniques such filtration, affinity binding or differential centrifugation.

In accordance with one embodiment, after incubation, reaction mixtures were centrifuged for 2 min at low speed (16,000g) and product procapsids

15   were then precipitated by addition of 1 ul of purified monoclonal antibody 6F10 (Spencer et al., Virol. 228:229-235 (1997)). Precipitates were allowed to form during 5 min incubation, at room temperature and then harvested by centrifugation at 16,000g for 30 sec. The precipitate was resuspended in 50 ul PBS and the procapsids dispersed by sonication before further operations were performed.

20   In accordance with the present invention procapsids can be formed readily from purified capsid proteins and in the absence of cells or cell extracts. The ability of procapsids to form without involvement of cell proteins is further suggested by the fact that only input virus proteins (and antibody) can be identified by SDS-polyacrylamide gel electrophoresis of the product procapsids. As with cell proteins,

25   cellular small molecules are also unlikely to play a major role in procapsid assembly from purified components. Purification of each of the three reaction components involves a desalting step that is expected to remove small molecules present in the original cell lysate.

Structural analyses by electron microscopy demonstrated a close

30   resemblance between procapsids assembled from purified components and those formed in Sf9 cell extracts. Like extract procapsids, those assembled from purified proteins were found to be spherical in overall shape with distinct shell and core layers.

Structural features of the shell, as revealed at 2.5 nm resolution in the three-dimensional reconstruction were indistinguishable from those of extract procapsids. Furthermore, the close structural similarity between the two procapsids is additionally noteworthy because different scaffolding proteins were employed in the two cases,

5   pre-VP22a in extract procapsids and the hybrid scaffold (pUL80.5-H) in purified protein procapsids. The structural similarity of the shells suggests that the identity of the scaffolding protein does not have a pronounced effect on shell morphology. Thus it is anticipated that other modified scaffolding proteins can be used to form capsids *in vitro* including scaffolding proteins modified to include a linked bioactive agent.

10   Based on the structural analysis of the capsids formed *in vitro*, there can be little doubt, that the structures formed from purified components are authentic procapsids and are representative structure of the natively formed capsids.

In accordance with one embodiment of the invention, the present *in vitro* system provides a model system for screening for therapeutic compositions that

15   inhibit HSV-1 capsid formation. Such therapeutic compositions can then be further investigated for their ability to prevent, cure or limit the symptoms of an HSV infection or other viral infection. As used herein the term "anti-herpes therapeutic" is used to refer to compounds that inhibit the replication of herpes virus and or prevent new infections by the herpes virus. In accordance with one embodiment, a method for

20   identifying anti-herpes therapeutics comprises combining purified proteins selected from the group consisting of VP5, VP23, VP19C and a viral scaffolding protein with a test composition to form a reaction mixture, wherein the test composition comprises one or more potential therapeutic compounds or agents. The mixture is then incubated at about 16°C to about 40°C to determine if capsid formation is prevented or

25   inhibited. As capsids are formed, the mixture will become increasingly turbid, and thus the rate of capsid formation can be monitored by measuring the amount of light deflected or absorbed by the mixture over time.

The scaffolding protein can be selected from the natural HSV-1 scaffolding proteins including pre-VP22a and VP22a, or the scaffolding protein can

30   be a derivative thereof such as the recombinant protein, pUL80.5-H. In accordance with one embodiment the therapeutic composition to be tested for anti-herpes activity can be added to the *in vitro* assembly at the beginning of the reaction. Alternatively

the purified HCV-1 proteins can be combined and pre-incubated for a preselected length of time so that procapsid formation is initiated before the addition of the test composition.

The present invention also encompasses a kit for conducting the *in vitro* assembly of HCV viruses. The kit would include each of the four purified proteins necessary for *in vitro* assembly (i.e. VP5, VP23, VP19C and the scaffolding protein), plus the appropriate buffered solutions for conducting the assembly of the capsids.

In accordance with one embodiment of the present invention the *in vitro* assembly system is used to prepare a delivery system for orally delivering a bioactive agent to the small intestine of vertebrates. More particularly, the formed capsids can be used as a delivery vehicle for delivering orally administered inactivation sensitive therapeutics through the acid environment of the stomach and into the small intestine where they can be absorbed into the bloodstream. The term "inactivation sensitive compound" and like terms as used herein is intended to include any bioactive compound that is inactivated or has reduced bioactivity after exposure to the conditions present in the upper portions of the alimentary tract, including the mouth, esophagus and stomach. Such compounds may be sensitive to proteases or to acidic conditions or a combination thereof. Bioactive compounds include any compound that is capable of inducing and effect on a living cell or organism, and include pharmaceuticals, hormones, chemotherapeutics, nucleic acids and the like.

The delivery system comprises an *in vitro* synthesized HSV viral capsid devoid of viral nucleic acids; and a bioactive agent entrapped within the viral capsid. Advantageously, the viral capsid is formed in the absence of eukaryotic cellular proteins and therefore the capsid delivery vehicle is devoid of such contaminants and consists essentially of viral proteins. In accordance with one embodiment a composition is provided for oral delivery of a bioactive agent to a vertebrate. The composition comprises a delivery vehicle consisting essentially of viral capsid proteins selected from the group consisting of VP5, VP23, VP19C and a viral scaffold protein, and a bioactive composition encapsulated by the delivery vehicle. Once the bioactive agent (therapeutic of choice) is introduced into the herpes simplex virus-1 (HSV-1) capsid, the capsids can be combined with a pharmaceutically

acceptable carrier and administered to a patient in need of the encapsulated therapeutic. The compositions can be administered intraparitoneally (for example intraveneously) or more preferrably the compositions are administered orally. The capsid provides a robust structure that gives considerable protection to the therapeutic

5   in the stomach environment. Degradation of the capsid in the small intestine then releases the drug for absorption.

Therapeutics can be introduced into the capsid in two ways, either by passive incorporation or by physically linking the therapeutic to a component of the capsid shell. Passive incorporation of therapeutic into capsids requires that the

10   therapeutic of choice (e.g. insulin) be included with the capsid proteins during the *in vitro* capsid assembly. In this method, the therapeutic is passively included in the finished capsid structure as illustrated schematically in Fig. 2.

Alternatively, the bioactive agent can be linked to one or more of the four capsid proteins in such a way that it is incorporated into the capsid as it is

15   assembled *in vitro*. In accordance with one embodiment the bioactive agent is linked to one of the capsid proteins via a protease sensitive linker. Suitable protease linkers are known to those skilled in that art. In one preferred embodiment, as shown in Fig. 3A, the bioactive agent (cargo) is linked to the scaffolding protein via a protease sensitive linker. The therapeutic is then released by enzymatic cleavage from the

20   hybrid molecule in the small intestine. In one preferred embodiment a therapeutic is linked to the scaffolding protein via a protease sensitive linker. One of the novel features of this method is the use of a hybrid molecule consisting of a capsid protein and the desired therapeutic for use in capsid assembly *in vitro*. Molecular genetic methods are employed to create a fusion of the capsid protein (say the scaffolding

25   protein) and the desired therapeutic. The two would be linked by a short peptide expected to be readily cleaved by proteases in the small intestine.

In the example shown in Fig. 3B, the linker is shown to consist of a series of lysine (K) residues which is expected to be cleaved by the enzyme trypsin. A requirement of the method is that the hybrid capsid-therapeutic molecule would need

30   to function normally in capsid assembly. The therapeutic would need to be small enough, and inert enough chemically that it does not interfere with *in vitro* capsid assembly.

Example 1

Formation of HSV-1 Capsids *In Vitro*

Materials and Methods

     Protein purification

5   All protein purification was initiated with Sf9 cells expressing the relevant herpes protein(s) after infection with an appropriate rBV vector or vectors. VP5 and pUL80.5-H were purified from cells infected with BAC-UL19 and BAC-UL80.5-H, respectively, while triplexes were purified from cells co-infected with BAC-UL18 and BAC-UL38. Construction of the four rBV and the methods

10   employed for their propagation and growth on Sf9 cells have been described previously (Oien et al., J. Virol. 71:1281-1291 (1997) and Thomsen and Homa J. Virol. 68:2442-2457 (1994). Protein purification was carried out beginning with cell lysates that were produced from pellets of rBV -infected cells. Cell pellets were diluted two-fold with PBS containing 20 mM EDTA and protease inhibitors

15   (complete, mini; Boehringer-Mannheim; 1 tablet/5ml) followed by three cycles of freezing and thawing to lyse the cells. Lysates were stored in 1 ml aliquots at -80°C until used. Lysate protein concentration was in the range of 15 mg/ml-20 mg/ml. Further purification steps were carried out at 4°C beginning with 2 ml of lysates that were thawed and clarified by centrifugation for 5 min at 16,000g.

20

Purification of VP5

                The clarified lysate was first subjected to centrifugation at 35,000 rpm (43,000g) for 30 min in a Beckman TL100 table top ultracentrifuge (TLA100.3 rotor). Sufficient saturated ammonium sulfate was then added to the resulting supernatant to

25   yield an ammonium sulfate concentration of 29% saturation. The mixture was incubated at 4°C for 30 min and then centrifuged at 16,000g for 10 min to collect the precipitate which contained VP5. The precipitate was dissolved in 3 ml PBS, 10 mM EDTA and desalted on a Bio-Rad EconoPac 10G desalting column (10 ml volume) eluted with 20 mM Tris-HCl pH 8. The sample was then filtered (Millipore; 0.22 um

30   pore size) and applied to a 1 ml Pharmacia Resource Q anion exchange column. The column was eluted with a 20 ml gradient of 0-1.0 M NaCl prepared in 20 mM Tris-HCl pH 8 and fractions (1.0 ml each) containing VP5, which eluted at approximately

0.3 M NaCl, were pooled.

VP5 from the column eluate was further purified by sucrose density gradient ultracentrifugation. A 0.5 ml aliquot of the Resource Q column eluate was applied to a 10%-30% linear sucrose gradient containing PBS plus 10 mM EDTA prepared in a 5 ml centrifuge tube. Gradients were centrifuged for 20 hr at 35,000 rpm in a Beckman LE 80K preparative ultracentrifuge (SW 50.1 rotor; 115,000g) operated at 4°C. The gradient was fractionated and VP5-containing fractions were identified by SDS-polyacrylamide gel electrophoresis. Relevant fractions were pooled, adjusted to 50% saturated ammonium sulfate to precipitate VP5 and centrifuged (35,000 rpm; SW50.1 rotor for 30. min) to recover the precipitate. The precipitate was dissolved in sufficient PBS, 10 mM EDT A (plus protease inhibitors) to yield a protein concentration of 1-2 mg/ml.

Purification of Triplexes

The clarified lysate of BAC-UL18/BAC-UL38-infected Sf9 cells was subjected to high speed clarification in the TL 100 ultracentrifuge, precipitated with ammonium sulfate, desalted and filtered as described above for purification of VP5 except that the ammonium sulfate precipitate was dissolved in 20 mM Tris-HCl pH 7. Triplexes were further purified by chromatography on a 1 ml Pharmacia Resource S cation exchange column. The sample was applied in 20 mM Tris-HCl pH 7 and eluted with a gradient of 0-1.0 MNaCl in the same buffer. Triplex-containing fractions, as identified by SDS-polyacrylamide gel electrophoresis, were pooled, precipitated with ammonium sulfate and dissolved in PBS, 10 mM EDTA as described above for VP5.

pUL80.5-H. Sf9 cell lysates containing pUL80.5-H (the hybrid scaffolding protein having a molecular weight of 39,855) were clarified by centrifugation at 16,000g for 15 min. Lysates were then adjusted to 29% saturated ammonium sulfate, incubated at 4°C and the precipitate collected as described above for VP5. The precipitate, which contained pUL80.5-H was dissolved in 0.5 ml PBS and fractionated further by sucrose density gradient ultracentrifugation. The sample was applied to the top of a 5.0 ml linear 10%-30%- sucrose gradient containing PBS prepared in a 5 ml Beckman Ultra-Clear SW 50.1 ultracentrifuge tube. Gradients

were centrifuged at 35,000 rpm (115,000g) in an SW 50.1 rotor for 4 hr at 4°C. After centrifugation, gradients were separated into 0.4 ml fractions which were examined by SDS-polyacrylamide gel electrophoresis for the presence ofpUL80.5-H. The desired fractions were pooled and pUL80.5-H precipitated by adjusting the solution to 50%

5   saturated ammonium sulfate. The precipitate was dissolved in sufficient PBS, 10 mM EDTA to yield a protein concentration of 2-3 mg/ml.

Procapsid assembly

Procapsids were assembled in reaction mixtures containing 10 ul VP5

10   (1-2 mg/ml), 10 ul triplexes (-1 mg/ml) and 10 Ul pUL80.5-H (2-3 mg/ml). Typical reaction mixtures therefore contained approximately 70 pmoles VP5, 84 pmoles triplexes and 500 pmoles pUL80.5-H. Reaction mixtures were adjusted to 25 mM EDTA, 10 mM DTT, and protease inhibitors were added before incubation for 1 hr at 37°C. After incubation, reaction mixtures were centrifuged for 2 min at low speed

15   (16,000g) and product procapsids were then precipitated by addition of 1 ul of purified monoclonal antibody 6F10 (4 mg/ml; 14,29). Precipitates were allowed to form during 5 min incubation, at room temperature and then harvested by centrifugation at 16,000g for 30 sec. The precipitate was resuspended in 50 ul PBS and the procapsids dispersed by sonication before further operations were performed.

20

Sucrose gradient analysis of purified proteins.

The oligomeric state of purified capsid proteins and protein complexes was analyzed by centrifugation on 0.7 ml sucrose density gradients. Linear 10%-30% sucrose gradients containing PBS were prepared in 5 mm X 41 mm (0.7 ml capacity)

25   Beckman Ultra-Clear ultracentrifuge tubes. A 20 ul sample (30 ul in the case of pUL80.5-H) of the specimen to be analyzed was adjusted to 25 mM EDTA, 10 mM DTT; appropriate protein standards were added (see below); and the mixture was layered on top of the gradient which was centrifuged in a Beckman SW50.1 rotor operated at 35,000 rpm. Centrifugation was for 14 hr-18 hr in the case of VP5 and

30   triplex specimens and 2.5 hr-4 hr in case of pUL80.5-H and VP5-pUL80.5-H complexes. Most gradients were centrifuged at 4°C although all specimens were also examined at 26°C and 34°C.

After centrifugation, gradients were separated into 14 equal fractions and an aliquot (20 ul) of each was analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining. Stained gels were scanned while wet on a flatbed scanner (Hewlett-Packard ScanJet IIc; reflected light), and bands were determined quantitatively by use of ImageQuant (Molecular Dynamics) software. The integrated density for individual bands was plotted as a function of gradient fraction number. The approximate molecular weights of proteins and protein complexes was determined with reference to protein standards by the use of the Martin and Ames treatment (Martin and Ames J. Biol. Chem. 236:1372-1379 (1961)): distance sedimented 1/distance sedimented 2 = $(MW_1/MW_2)^{2/3}$. Protein standards employed were: bovine serum albumin (MW 68,000); B-amylase (MW 200,000); and apo-ferritin (MW 443,000).

Cryo-electron microscopy.

Freshly assembled procapsids in PBS were concentrated by adding the monoclonal antibody 6F10 (Spencer et al., Virol. 228:229-235 (1997)), and the resulting precipitate was prepared for cryo-electron microscopy by adsorption to a continuous thin carbon film supported on a thick holey carbon film. The drop was blotted to a thin film, quenched in liquid ethane cooled by liquid nitrogen in a Reichert FC4 cryo-station, transferred into a Gatan-626 cryo-holder, and observed on a Philips CM200-FEG electron microscope, as described by Zlotnick et al., Biochem. 35:7412-7421 (1996). Micrographs were recorded at a nominal magnification of 38,000x using minimal electron dose techniques, producing radiation levels of about 8 electrons/ $Å^2$.

Image processing.

A three-dimensional reconstruction of the procapsid was computed beginning with micrographs that were selected for analysis by visual appraisal (e.g. to assess density of particles and contrast) and by optical diffraction to assess the state of defocus and resolution. Four micrographs whose first CTF zeroes were in the range of 1/22 Å to 1/24 Å were scanned at 26 um/pixel on a Perkin Elmer 1010 MG microdensitometer yielding an effective pixel size of about 7 Å. A total of 94 capsid

images were processed as previously described (Trus et al., J. Mol. Biol. 263:447-462 (1996)). The structure was solved using the PFT method (Baker and Cheng J. Struct. Biol. 116:120-130 (1998)), starting with our earlier result for extract-assembled procapsids as a starting model. As a control against imprinting the features of the

5   starting model on the reconstruction, the calculation was repeated using a density map of the mature B-capsid (Conway et al., J. Struct. Biol. 116:200-208 (1996)) as starting model. Identical results were obtained. After iterative cycles of refinement of orientation angles and origins, the 72 particles with the highest correlation coefficients were selected and a density map was calculated to 25 Å resolution, as assessed by the

10   FRC3D criterion.

Other methods

     Procapsid-containing precipitates were prepared for thin section electron microscopy by fixation, embedding in Epon 812 and sectioning as previously

15   described (Matusick-Kumar et al., J. Virol. 68:5384-5394 (1994) and Newcomb et al., J. Mol. Biol. 263:432-446 (1996)). For negative stain electron microscopy, specimens were stained with 1% (w/v) uranyl acetate as described by Thomas et al. (Thomas, et al., J. Virol. 54:598-607 (1985)). All thin section and negative stain electron micrographs were recorded on a JEOL 100CX transmission electron microscope

20   operated at 80keV. SDS-polyacrylamide gel electrophoresis was carried out on 6 cm X 9 cm slabs of 12% polyacrylamide. Protein bands were identified by Coomassie blue staining and determined quantitatively by scanning as described above. Scans were taken in the dynamic range of the scanner with the error of the measurement estimated to be +/-5%.

25   Western immunoblotting was performed with similar gels in which proteins were transferred electrophoretically to Immobilon membranes and stained as described by Spencer et al. (Spencer et al., Virol. 228:229-235 (1997)). Specific staining was carried out with: (1) rabbit polyclonal antisera specific for VP5, VP19C or VP23 (generously donated by Drs. G. Cohen and R. Eisenberg); or (2) a rabbit

30   polyclonal antiserum specific for the HCMV scaffolding protein (UL80.5 gene product; see Oien, et al., J. Virol. 71:1281-1291 (1997)). All specific antisera were used at a 1:5000 dilution. HSV-1 B-capsids used as reference standards in SDS-

polyacrylamide gel electrophoresis and immunoblotting studies were prepared from BHK-21 cells infected with the 17MP strain ofHSV-1 as previously described (Newcomb, et al., J. Mol. Biol. 232:499-511 (1993)).

5   RESULTS

Proteins employed for procapsid assembly

Procapsid assembly was carried out with VP5, triplexes (composed of VP19C and VP23) and a hybrid scaffolding protein called pUL80.5-H (MW 39,855; see Oien, et al., J. Virol. 71:1281-1291 (1997)). The latter consists of the N-

10   terminal364 amino acids of the human cytomegalovirus (HCMV) scaffolding protein (UL80.5 gene) linked to the C-terminal 25 amino acids of the corresponding HSV-1 protein (pre-VP22a; UL26.5 gene). Although assembly of HSV-1 procapsids was accomplished with the endogenous HSV-1 scaffolding protein in unpurified form in cell extracts (Newcomb, et al., J. Mol. Biol. 263:432-446 (1996)), yields of this

15   protein in subsequent attempts at expression and purification were insufficient for the current project. Since procapsids have also been assembled with the hybrid scaffolding protein *in vivo* and *in vitro* from cell extracts, and this protein proved more tractable in purification trials, the hybrid scaffolding protein was used throughout the project. All proteins were purified from Sf9 cells expressing the

20   relevant herpes protein(s) as a result of infection with an appropriate rBV vector or vectors. In each case, protein purification began with a clarified cell lysate produced as described in the Materials and Methods.

VP5 was purified by ammonium sulfate precipitation followed by anion exchange chromatography and sucrose density gradient centrifugation. After

25   each stage of purification VP5-containing fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Densitometric scanning of the stained gels was employed to estimate the amount of VP5 as a proportion of the total protein present and the results are shown in Table 1.

TABLE 1. Purification summary for VP5, triplexes and pUL80.5-H

Purification Step	% of Total Protein Present[a]		
	VP5	Triplex	pUL80.5-H
1. Clarified Lysate	17%	11%	24%
2. Ammonium Sulfate Precipitation	41%	48%	46%
3. Anion Exchange Chromatography	79%	NA	NA
4. Cation Exchange Chromatography	NA	95%	NA
5. Sucrose Density Gradient	96%	NA	93%

[a] The percent of each capsid protein present was determined by densitometric scanning of a Coomassie-blue-stained SDS-polyacrylamide gel.,

NA: Not applicable

As both gel and densitometric analyses show, VP5 represented quite a high fraction (17%) of the original clarified lysate indicating that it was expressed at a high level from the BAC-UL19 vector and that it accumulated in a soluble form. Ammonium sulfate precipitation and anion exchange chromatography resulted in enrichments of approximately 2.5-fold and 2-fold, respectively. The anion exchange column eluate had a prominent VP5-containing peak which was resolved from bands of cell proteins eluting at lower and higher NaCl concentrations, respectively. The sucrose density gradient step separated VP5 from several proteins migrating between VP5 and VP23 resulting in a product in which VP5 accounted for 96% of the protein present as shown in Table 1. Western immunoblot analyses of VP5-containing fractions demonstrated that the gel band identified as VP5 was able to react with an antiserum specific for VP5 after each of the three purification steps).

Triplexes were purified from Sf9 cells co-infected with rBV encoding VP19C and VP23. The goal was to isolate triplexes as a unit since previous studies had shown that triplexes are stable in Sf9 cell extracts (Spencer, et al., J. Virol. 72:3944-3951 (1998)). Purification was accomplished by ammonium sulfate

precipitation followed by cation exchange chromatography, the two steps resulting in enrichments of approximately 4-fold and 2-fold, respectively as shown in Table 1. The success of the cation exchange chromatography step appeared to be due in part to the fact that many non-triplex proteins in the ammonium sulfate precipitate did not

5 bind to the column. Only small amounts of non-triplex proteins were recovered in the column eluate. The molar ratio of VP23 : VP 19C in the purified triplexes was determined to be 1.7 (average of three preparations) from quantitative analysis of stained bands on SDS-polyacrylamide gels. We interpret this as agreeing satisfactorily with 2.0, the expected ratio for heterotrimeric virion triplexes. The

10 bands identified as VP 19C and VP23 in purified triplex preparations were shown to react with polyclonal antisera specific for the two proteins.

The scaffolding protein pUL80.5-H was purified from the clarified lysate by ammonium sulfate precipitation followed by sucrose density gradient ultracentrifugation as described in the Materials and Methods. During sucrose density

15 gradient centrifugation pUL80.5-H migrated as a very broad band from the top of the gradient nearly to the bottom. The most rapidly migrating material was found in a peak whose sedimentation rate (compared to protein standards) suggested a structure with a molecular weight of approximately 1×10^6. This most rapidly-migrating material was collected from the gradient and employed for-capsid assembly studies.

20 Electron microscopic analysis of negatively stained specimens showed that this material consists of roughly spherical, variably sized particles with a diameter of approximately 28 nm. For example, measurement of 39 particles yielded an average diameter (longest dimension) of 28 ± 4 nm. In a Western immunoblot, purified pUL80.5-H was found to react with a polyclonal antibody specific for pUL80.5-H .

25

Procapsid assembly

Procapsids were formed by mixing VP5, triplexes and pUL80.5-H together and incubating as described in Materials and Methods. The order of component addition did not appear to affect the quality or quantity of procapsids

30 formed. During the incubation period, the reaction mixture became visibly turbid as procapsids were assembled. Turbidity, presumed to be due to procapsids, could not be removed by low speed centrifugation (16,000g) at this stage. Procapsid formation

required incubation at temperatures of approximately 26°C or higher. No procapsids formed if reaction components were maintained at 4°C. After harvesting by antibody precipitation, procapsids were examined by electron microscopy of frozen hydrated, thin-sectioned and negatively stained preparations. Frozen hydrated specimens

5   appeared uniform in morphology and round in profile suggesting the procapsids are spherical. The measured diameter was 126 +/-1 nm (n =12). Distinct shell and core layers were visible with a region of lower density between the two. A region of low density was seen at the center of nearly all images. Capsomers could often be resolved at the periphery.

10   Distinct shell and core layers were also observed in thin-sectioned preparations. In most images the core stained somewhat more darkly than the shell, and weakly staining regions were found at the center of the image and between the shell and core layers. Thin-sectioned preparations showed that procapsids formed from purified proteins were similar in structure to procapsids formed in vitro from Sf9

15   cell extracts containing HSV-1 proteins.

Surface features of the procapsid were emphasized in images of negatively stained specimens. Capsomers were visible both en face at the center of images and in profile at the procapsid periphery. As in the case of thin-sectioned specimens, negatively stained procapsids assembled from purified proteins resembled

20   their counterparts prepared from cell extracts. The round morphology observed for procapsids contrasts with the angular, icosahedral shape seen in mature HSV-1 B-capsids.

A three-dimensional reconstruction of the procapsid was computed beginning with cryo-electron micrographs. A total of 72 procapsid images were

25   employed, and the reconstruction was calculated to a resolution of 25 Å using the PFT method (Baker, et al., J. Struct. Biol. 116:120-130 (1998)). For comparison, The reconstruction computed previously for procapsids formed from HSV -1 proteins in Sf9 cell extracts was compared to capsids prepared in accordance with the present invention. Comparison of the two reconstructions shows that procapsids prepared in

30   the two ways are closely similar in nearly all respects. For example, both kinds of procapsids are spherical in shape and have holes through the capsid wall that do not exist in the mature capsid. In both cases, P-hexons and E-hexons (30) are oval in

shape. This asymmetry is not expressed on the inner surface of the procapsid shell where each capsomer has a continuous rim of density that is hexagonal for hexons and pentagonal for pentons.

A minor difference between the two procapsids was observed in the morphology of the core. In central thin sections it is apparent that the cores of the two kinds of procapsids differ in their radial density. In particular, the core of the extract-assembled procapsid, which is composed primarily of pre-VP22a, has a sharply defined dense ring at a radius of about 270 Å. This ring is absent from the pUL80.5-H-containing core of the procapsids assembled from purified proteins. The cores appear to be comparably well preserved in the two kinds of procapsids so the above differences relate to the respective substructures of the two scaffolding proteins.

Analysis of the procapsid protein composition by SDS-polyacrylamide gel electrophoresis showed that all four input proteins were present in the product procapsids. Except for the scaffolding protein, their relative proportions were similar to the proportions found in B-capsids. In a representative experiment, for example, the proportions of VP5:VP19C: scaffold:VP23 as measured by densitometric analysis of a stained gel were 1.00 : 0.24 : 1.23 : 0.37 for the procapsid and 1.00 : 0.31 : 0.45 : 0.35 for B-capsids. The higher amount of scaffolding protein present in procapsids compared to B-capsids was also observed earlier with procapsids formed in cell extracts.

As an overall measure of the efficiency of procapsid assembly, we determined the proportion of VP5 that was incorporated into the antibody-precipitable procapsid fraction. VP5 was employed for this determination because it is the reaction component present in limiting quantity. Reaction mixtures were constituted, incubated and precipitated with mAb-6F 10 as described in Materials and Methods. The precipitate was harvested by low-speed centrifugation, and samples of both precipitate and supernatant were analyzed by SDS-polyacrylamide gel electrophoresis. Densitometric scanning of the stained gel showed that 88% of the input VP5 was found in the precipitate and 12% in the supernatant. In contrast, less than 5% of the input VP5 was found in the precipitate if pUL80.5-H was omitted from the reaction mixture.

Oligomeric state of purified components

The oligomeric state of purified procapsid components was examined by sucrose density gradient ultracentrifugation. Samples of purified components were mixed with appropriate protein standards, centrifuged on sucrose gradients, and the gradients were separated into equal fractions as described in Materials and Methods. The positions of procapsid proteins in the gradient was determined by SDS-polyacrylamide gel electrophoresis of gradient fractions followed by densitometric scanning of the stained gel. The integrated density corresponding to each protein was then plotted as a function of fraction number. VP5 was found to sediment between the serum albumin (MW 68,000) and B-amylase (MW 200,000) markers suggesting it is a monomer under the conditions employed. Only a trace of VP5 was found in material sedimenting more rapidly than B-amylase where any dimer or higher oligomer is expected to be found. The sedimentation behavior of VP5 was not strongly affected by protein concentration over the range of 0.1 mg/ml to 1.5 mg/ml or by temperature in the range of 4°C to 26 °C.

VP 19C and VP23 were found to sediment together in a single band between the BSA and B-amylase (not shown) markers. The molecular weight of the triplexes as estimated with reference to the protein standards was 107,000, a value in satisfactory agreement with the molecular weight of 118,796 expected of a structure containing one VP 19C plus a dimer of VP23. Gradients showed no evidence of structures migrating more slowly than the triplex band. As in the case of VP5, sedimentation of the triplexes showed little dependence on protein concentration in the range of 0.5 mg/ml-1.5 mg/ml or temperature (4°C- 34° C).

Gradient analyses indicated that the oligomeric state of pUL80.5-H was strongly dependent on protein concentration. For example, when analyses were performed as described above with 30 ul samples containing 5 ug, 25 ug and 75 ug of pUL80.5-H (low, medium and high concentrations, respectively) different results were obtained. At low concentration, pUL80.5-H migrated as a broad distribution extending from the top of the gradient to approximately fraction six with a peak at fraction four. The distribution was even broader at medium and high concentration extending from the top of the gradient to fractions 11-13 at the highest concentration tested. The molecular weights of the pUL80.5-H complexes, when calculated with

respect to BSA, B-amylase and apo-ferritin standards, were found to extend from the monomer up to a MW of approximately 1 X 10⁶ or more corresponding to oligomers containing 20-30 or more pUL80.5-H molecules. The complexes found at fractions 12-13 correspond in their sedimentation rate to the material harvested in the final step

5   of pUL80.5-H purification. pUL80.5-H did not sediment more rapidly than the fraction 12-13 band at any concentration tested. When material from the fraction 12-13 band was diluted and re-centrifuged, its migration was characteristic of the new, lower concentration, suggesting the distribution ofpUL80.5-H oligomers re-equilibrates readily. The sedimentation behavior ofpUL80.5-H was not strongly

10 influenced by temperature in the range of 4°C-34°C.

VP5-pUL8O.5-H complexes

Sucrose gradient ultracentrifugation was also employed to examine complexes formed between VP5 and pUL80.5-H. Analysis was carried out at 4°C as

15 described above for purified proteins except that VP5 and pUL80 5-H were mixed prior to centrifugation. All experiments were carried out at a molar excess of pUL80.5-H over VP5, with pUL80.5-H:VP5 ratios in the range of 10: 1 to 2: 1. Results were obtained with two specimens containing pUL80.5-H and VP5 at molar ratios of 4: 1 and 8: 1, respectively. In both cases pUL80.5-H was found to migrate as

20 a broad band extending across most of the gradient as it did in the absence ofVP5. Migration of VP5, however, was found to be affected by the presence of pUL80.5-H indicating an interaction between the two proteins. At a 4: 1 ratio of pUL80.5-H:VP5, VP5 migrated as a band corresponding to a complex with an estimated MW of200,000-270,000 which was distinct from the band of VP5 sedimented in the

25 absence ofpUL80.5-H. The 200,000-270,000 band was also observed at 8: 1 pUL80.5-H:VP5, but in this case the VP5 distribution extended further down the gradient suggesting the existence of larger complexes, the largest corresponding to an estimated MW of about 500,000-600,000. Only trace amounts ofVP5 were found to migrate co-incidentally with the major MW 1 X 10⁶ pUL80.5-H band. No VP5-

30 containing complexes were found outside the region between approximately fractions 3 and 9 under any experimental conditions tested.

DISCUSSION

Protein purification.

It was attractive to initiate protein purification beginning with extracts of rB V -infected Sf9 cells because capsid proteins were known to be competent for procapsid assembly in such preparations. VP19C and VP23 were purified as a complex from cells co-infected with rBV encoding both proteins rather than as separate species for three reasons: First, it was observed that VP19C and VP23 participate in assembly only after they associate with each other to form triplexes; neither protein can bind to nascent capsids without the other. Second, pre-formed triplexes were found to remain intact and to retain their assembly competence during purification. Third, attempts to purify assembly competent VP 19C from Sf9 cells containing it were unsuccessful. In extracts, VP 19C proved sensitive to proteolytic digestion and the purified protein did not support procapsid formation, however association of VP 19C with VP23 is believed to stabilize VP 19C against proteolysis or other processes that denature or inactivate it.

Purification of pUL80.5-H by sucrose density gradient ultracentrifugation as described here depended critically on its presence in the form of large (28 nm diameter) oligomers. When the ammonium sulfate-fractionated lysate containing pUL80.5-H was centrifuged on sucrose gradients, the large scaffolding protein oligomers migrated more rapidly than contaminating cellular proteins affording a substantial enrichment in pUL80.5-H. Similar sucrose gradient analyses of the HSV -1 scaffolding protein, pre-VP22a, have shown that it also forms irregularly-shaped oligomers with approximately the same size and shape as those formed by pUL80.5-H.

Protein oligomeric state

Sucrose density gradient analysis of purified VP5 demonstrated that it migrated between the BSA and B-amylase markers suggesting it is a monomer in solution. No larger structures were observed at any concentration tested. Since monoclonal antibodies cannot ordinarily precipitate protein monomers, the presence of VP5 as a monomer in solution accounts for the observation that it is not precipitated by specific monoclonal antibodies such as 6F10 unless it is first

complexed with scaffolding or scaffolding plus triplex proteins.

Analyses of purified triplexes on sucrose gradients were in agreement with earlier studies of triplexes in Sf9 cell extracts. The two triplex proteins were found to migrate together in a structure with a composition and estimated molecular

5   weight compatible with a heterotrimer consisting of one VP 19C and two VP23 molecules. Gradients containing triplexes were remarkable for the fact that they showed little or no evidence for dissociation of triplexes into individual proteins suggesting a very strong association between VP19C and VP23.

The oligomeric state of pUL80.5-H as determined by sucrose gradient

10   analysis was found to be strongly dependent on protein concentration. Larger structures were favored at higher protein concentrations. The largest were estimated on the basis of their sedimentation rate to correspond to oligomers of 25-30 pUL80.5-H molecules (i.e. the 28 nm diameter particles) while the smallest, found near the tops of the gradients, corresponded to monomers or dimers. The broad range of pUL80.5-H

15   oligomers observed by sucrose gradient analysis were interpreted to be due to the ability of pUL80.5-H to self-associate in many different proportions in an equilibrium in which larger oligomers are favored by higher protein concentration.

It was expected that pUL80.5-H would be found to self-associate as studies involving the yeast two-hybrid system have demonstrated self interactions in

20   both HCMV and HSV -1 scaffolding proteins. Specific regions involved in self-interaction have been identified in both cases, and an α-helical, coiled-coil motif has been proposed for the HSV-1 pre-VP22a self association. In both HCMV and HSV-1, the ability of the scaffolding protein to self-associate has been found to be required for its interaction with the major capsid protein. The importance of scaffold self-

25   association for procapsid formation is emphasized by the observation that VP5 molecules do not interact in the absence of a scaffolding protein. It suggests that either: (a) binding of VP5 to a scaffolding protein causes VP5 to acquire the ability to self associate; or (b) procapsid formation involving VP5-scaffolding protein complexes depends critically on scaffold-scaffold interactions to concentrate VP5

30   molecules in the proper physical relationship to each other forming loosely associated precursor capsids that become more tightly bound when angularization takes place.

Procapsid assembly

Procapsids formed readily when purified VP5, triplexes and pUL80.5-H were mixed and incubated. The ability of procapsids to assemble from purified proteins indicates that cell-encoded proteins are not required for assembly. The ability

5   of procapsids to form without involvement of cell proteins is further suggested by the fact that only input virus proteins (and antibody) can be identified by SDS-polyacrylamide gel electrophoresis of the product procapsids. As with cell proteins, cellular small molecules are also unlikely to play a major role in procapsid assembly from purified components. Purification of each of the three reaction components

10   involves a desalting step that is expected to remove small molecules present in the original cell lysate.

Electron microscopic analysis of capsids assembled from purified proteins demonstrated that nearly all were round, not angular in profile suggesting they correspond to the spherical procapsid rather than to the mature, icosahedral

15   capsid. The proportion of angular capsids was less than approximately 15%, when incubations were carried out for 4 hrs at 37°C. Reaction mixtures containing purified proteins differ significantly in this respect from assembly in Sf9 cell extracts where nearly all capsids are angular after 2 hrs or more of incubation at 37°C. Possible explanations for the lower number of angular capsids in the purified system include:

20   (a) use of the hybrid HCMV -HSV-1 scaffold rather than the homologous HSV -1 form; (b) the potential role for a cell-encoded protein (e.g. a protease) in angularization; and (c) the possibility that ions or other small molecules required for angularization were not present in reaction mixtures.

Structural analyses by electron microscopy demonstrated a close

25   resemblance between procapsids assembled from purified components and those formed in Sf9 cell extracts. Like extract procapsids, those assembled from purified proteins were found to be spherical in overall shape with distinct shell and core layers. Structural features of the shell, as revealed at 2.5 nm resolution in the three-dimensional reconstruction were indistinguishable from those of extract procapsids.

30   There can be little doubt, therefore, that the structures formed from purified components are authentic procapsids. The close structural similarity between the two procapsids is additionally noteworthy because different scaffolding proteins were

employed in the two cases, pre-VP22a in extract procapsids and the hybrid scaffold (pUL80.5-H) in purified protein procapsids. The structural similarity of the shells suggests that the identity of the scaffolding protein does not have a pronounced effect on shell morphology.

5   In accordance with one embodiment a compound can be linked to the scaffolding protein to ensure that sufficient quantities of the compound are loaded into the capsid shell upon its formation *in vitro*.

           Like procapsids formed in cell extracts, those assembled from purified components were sensitive to disruption at 2°C. The protein composition of

10   procapsids, as determined by SDS-polyacrylamide gel analysis, demonstrated that procapsids contain all four herpes proteins added. Their relative proportions were found to be similar to those of B-capsids except for the scaffolding protein which was regularly observed to be higher in procapsids. The higher amount of scaffold in the procapsid may result from its presence in immune precipitates in a form other than

15   procapsids. This possibility was addressed by analyzing the procapsid reconstruction. Density corresponding to the shell and core layers were integrated separately and found in the ratio of 2.62 shell: 1 core. Assuming the core is entirely pUL80.5-H, this ratio indicates a pUL80.5-H copy number of 1736, a value significantly higher than the 1153 +/- 169 reported for B-capsids.

20   The efficiency of procapsid formation as estimated from the proportion of input VP5 incorporated into material precipitated by mAb-6F10 (88%) represents an upper limit to the procapsid assembly yield. In addition to completed procapsids, mAb-6F10 also precipitates VP5-scaffolding complexes that may be formed in addition to procapsids in reaction mixtures. Analysis of procapsid precipitates by thin

25   section electron microscopy demonstrated the presence of non-procapsid material that could correspond to VP5-pUL80.5-H complexes. Less non-procapsid material was observed, however, in negatively-stained and frozen hydrated preparations suggesting that procapsids account for a high proportion of the overall antibody precipitate.

30   VP5-scaffolding protein complexes

           Sucrose gradient analyses of VP5-pUL80.5-H mixtures demonstrated that the two proteins interact readily to form complexes with estimated molecular

weights in the range of 200,000-600,000. Taking into account the protein composition of the complexes, we interpret them to be a population of oligomers in which individual species contain one or two VP5 and 1-5 or 6 pUL80.5-H molecules. Little or no free VP5 was observed in the gradients suggesting that interaction between the two proteins is strong enough that complexes do not dissociate during sucrose gradient centrifugation.

The VP5-pUL80.5-H complexes observed by gradient analysis suggest themselves as functional subunits involved in procapsid assembly. Complexes could be cross-linked to each other by way of the triplexes as shown diagramatically in Fig. 1 to initiate or extend growth of the nascent procapsid. Involvement of VP5-pUL80.5-H complexes in procapsid formation is consistent with the observation that procapsids appear to form by incremental addition of both VP5 and scaffolding protein to partial capsid intermediates. The VP5-scaffolding protein complexes observed here could suggest the form in which VP5 and pre-VP22a are transported to the nucleus in HSV -1-infected cells. Although VP5 can enter the nucleus in other ways, VP5 and pre-VP22a can enter the nucleus as a complex, and those described here are small enough that they should be able to be transported through nuclear pores. A consistent feature of the sucrose gradient analyses was the observation that VP5 interacted weakly or not at all with the large (MW 1.1 X 106; 28 nm diameter) pUL80.5-H particles. If they occur in infected cells, therefore, the large particles are likely to serve as a reservoir of scaffolding protein rather than as direct participants in the assembly process.

Example 2

Capsid Loading

To determine the efficiency of loading the HCV capsids with a therapeutic compositions the following test will be conducted. Tests of capsid loading and delivery will be performed separately with tests of loading being done first. Loading will be assayed initially with hemoglobin (Hb) and with green fluorescent protein (GFP). The loading of these two proteins is selected because they are easily detected due to their red color (Hb) and strong fluorescent signal (GFP), respectively. *In vitro* capsid assembly will be performed in the presence of Hb or GFP. The resulting capsids will then be harvested by antibody precipitation and tested for the

presence of Hb or GFP. Their presence will be taken as evidence the protein was incorporated into capsids during their formation. Control experiments will be carried out to verify that encapsidation requires capsid formation *in vitro* and that encapsidated proteins are found inside, and not outside, the capsid shell.

5   Similar experiments will be carried out with constructs in which GFP is covalently linked to the HSV-1 scaffolding protein, pre-VP22a. Linkage will be by way of a protease-sensitive linker as described above. The gene encoding the hybrid molecule will be produced by standard molecular cloning methods, and expressed in an appropriate host system such as *E. coli* or insect cells infected with a recombinant

10   baculovirus. Standard biochemical procedures (W. W. Newcomb et al., Journal of Virology Vol. 73, pg. 4239-4250 (1999)) will be employed to purify the fusion protein for use the *in vitro* capsid assembly system.

Example 3

15   **Drug Delivery**

Drug delivery requires encapsidation followed by passage of the encapsidated therapeutic through the stomach and release of the drug in the small intestine. To test the ability of the loaded capsids of the present invention to deliver an effective amount of an inactivation sensitive therapeutic to the intestines, the capsids

20   will be loaded with insulin and administered orally to a patient. Insulin will be encapsidated either passively or by way of a fusion protein into capsids formed *in vitro* and fed to mice whose blood glucose levels will be tested before and after feeding encapsidated insulin. Blood glucose levels will be measured with a standard glucometer available in our laboratory. A significant decrease in blood glucose level

25   will be regarded as evidence encapsidated insulin was successfully delivered to the small intestine. Control experiments will be carried out to test capsids lacking insulin and free insulin not contained in capsids.

Claims:

1.   A method for identifying anti-herpes therapeutics, said method comprising

combining purified proteins selected from the group consisting of VP5, VP23, VP19C and a viral scaffold protein with a test composition to form a reaction mixture, said test composition comprising a potential therapeutic compound or agent;

incubating the reaction mixture at about 16°C to about 40°C;

determining the turbidity of the reaction mixture.

2.   The method of claim 1 wherein the scaffold protein is pre-VP22a.

3.   The method of claim 1 wherein the scaffold protein is VP22a.

4.   The method of claim 1 wherein the purified proteins are combined and pre-incubated for a preselected length of time before addition of the test composition.

5.   A kit for testing the therapeutic effectiveness of an anti-herpes composition, said kit comprising

purified proteins selected from the group consisting of VP5, VP23 VP19C and a viral scaffold protein.

6.   A delivery system for orally delivering a bioactive agent to the small intestine of vertebrates, said delivery system comprising

an in vitro synthesized viral capsid devoid of viral nucleic acids; and

a bioactive agent entrapped within the viral capsid.

7.   The delivery system of the claim 6 wherein the bioactive agent is linked to one or more of the capsid proteins

8.   The delivery system of the claim 6 wherein the bioactive agent is linked to the scaffold protein.

9.   The delivery system of claim 8 wherein the bioactive agent is covalently bound to the scaffold protein via a protease sensitive linker.

10.   A method for preparing a viral capsid delivery system, said method comprising the steps of

combining purified proteins selected from the group consisting of VP5, VP23 VP19C and a viral scaffold protein with a bioactive agent to form a reaction mixture;

incubating the reaction mixture at about 16°C to about 40°C; and
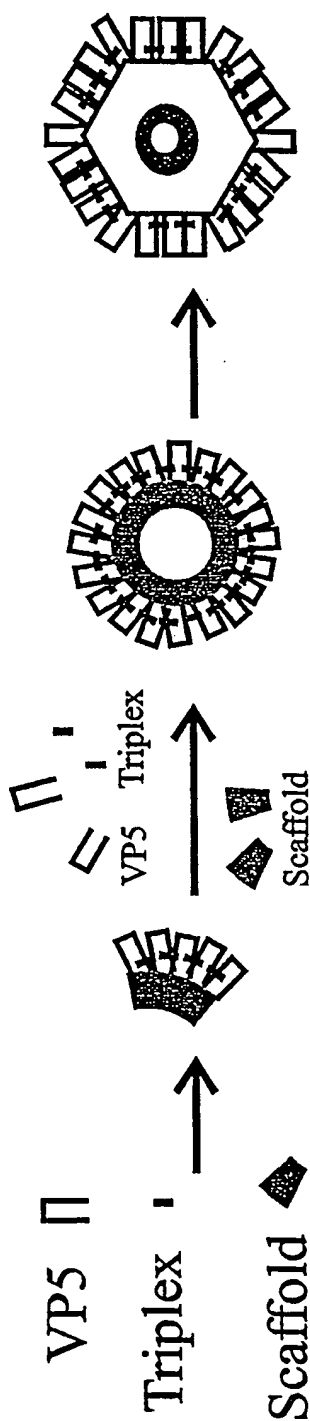
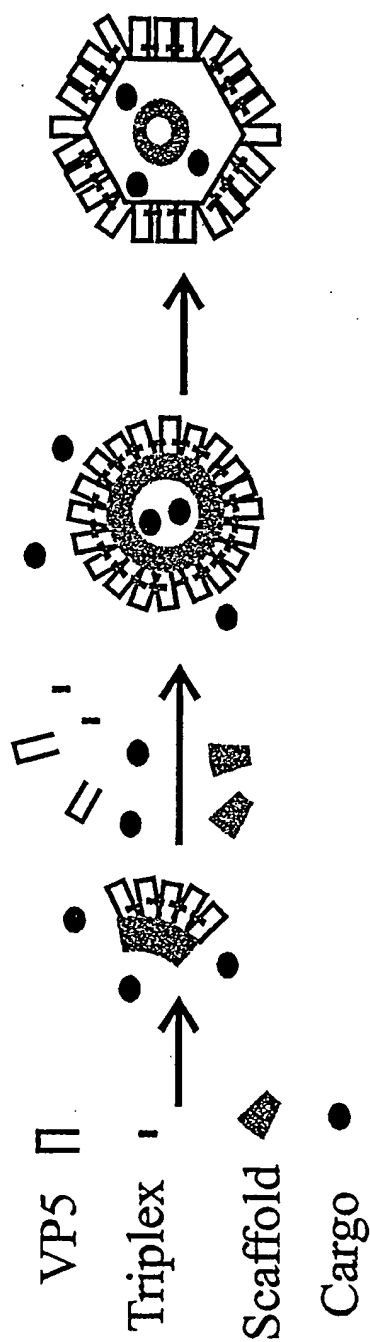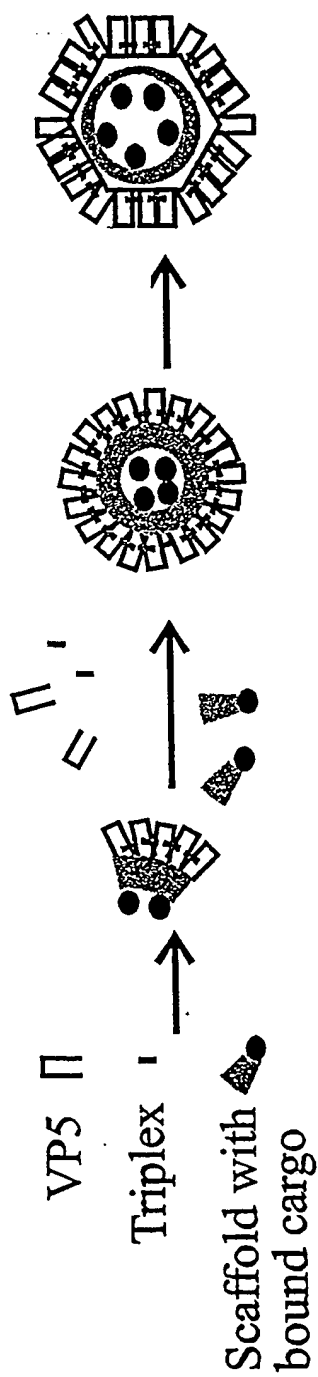recovering the mature capsids.

Figure 1

VP5

Triplex

Scaffold
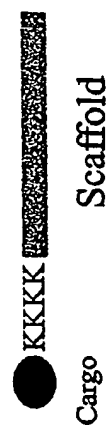
Cargo

Figure 2

VP5

Triplex

Scaffold with
bound cargo

Figure 3a

KKKK Scaffold

Cargo

Figure 3b

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/245, 39/12; C12Q 1/70
US CL : 424/229.1, 204.1; 435/5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/229.1, 204.1; 435/5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, WEST
search terms: HSV?, VP19C, VP23, VP5, ICP35, VP22a, cell free system, diagnos?; kit

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NICHOLSON et al. Localization of herpes simplex virus type 1 major capsid protein VP5 to the cell nucleus requires the abundant scaffolding protein VP22a. Journal of General Virology. 1994, Vol. 75, pages 1091-1099, especially page 1098.	1-10
Y	THOMSEN et al. Assembly of Herpes Simplex Virus (HSV) Intermediate Capsids in Inset Cells Infected with Recombinant Baculoviruses Expressing HSV Capsid Proteins. Journal of Virology. April 1994, Vol. 68, No. 4, pages 2442-2457, see the entire document.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier document published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 DECEMBER 1999	23 DEC 1999

Name and mailing address of the ISA/US	Authorized officer
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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NEWCOMB et al. Cell-Free Assembly of the Herpes Simplex Virus Capsid. Journal of Virology. September 1994, Vol. 68, No. 9, pages 6059-6063, see the entire document.	1-10
Y	MATUSICK-KUMAR et al. The C-Terminal 25 Amino Acids of Protease and Its Substaret ICP35 of Herpes Simplex Virus Type 1 Are Involved in the Formation of Sealed Capsids. Journal of Virology. July 1995, Vol. 69, No. 7, pages 4347-4356, see the abstract.	1-10
Y	PELLETIER et al. Self-Association of Herpes Simplex Virus Type 1 ICP35 Is via Coiled-Coil Interactions and Promotes Stable Interaction with Major Capsid Protein. Journal of Virology. July 1997, Vol. 71, No. 7, pages 5197-5208, see the abstract.	1-10
Y	OIEN et al. Assembly of Herpes Simplex Virus Capsids Using the Human Cytomegalovirus Scaffold Protein: Critical Role of the C Terminus. Journal of Virology. February 1997, Vol. 71, No. 2, pages 1281-1291, see the entire document.	1-10
Y	SPENCER et al. Assembly of the Herpes Simplex Virus Capsid: Preformed Triplexes Bind to the Nascent Capsid. Journal of Virology. May 1998, Vol. 72, No. 5, pages 3944-3951, see the abstract.	1-10